

(12) PATENT ABSTRACT (11) Document No. AU-A-21725/88
(19) AUSTRALIAN PATENT OFFICE

(54) Title
CONJUGATES OF CYTOKINES WITH IMMUNOGLOBULINS

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(51)* International Patent Classification(s)
C07K 015/12 C07K 015/26 A61K 037/02

(21) Application No. : 21725/88 (22) Application Date : 01.09.88

(30) Priority Data

(31) Number (32) Date (33) Country
3357/87 02.09.87 CH SWITZERLAND

(43) Publication Date : 23.3.89

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(57) Claim

1. Conjugates of cytokines with human immunoglobulin.
2. Conjugates according to claim 1 of the formula

Ck-Z-Ig (I)

in which Ck is a residue of a natural human cytokine or of a recombinant cytokine, Ig is a residue of a human immunoglobulin and Z is a covalent bond or a covalently bonded organic bridge-former, and in which Ck, Ig and/or Z also may occur several times.

3. Conjugates according to claim 2 of the formula I in which Ck is a residue of a natural or recombinant human interferon, of interleukin, of a related compound or of a fragment that retains the biological activity, Ig is a residue of a natural human immunoglobulin, Z is a covalent bond or a covalently bonded di-, tri- or tetra-valent hydrocarbon radical which, if desired, is substituted and/or in which, if desired, one or more carbon atoms have been replaced by oxygen, sulfur and/or unsubstituted or substituted nitrogen atoms, and in which Ck, Ig and/or Z also may occur several times.

9. A process according to claim 8 for the preparation of conjugates of the formula

Ck-Z-Ig (I),

12

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-2-

which comprises so reacting 2 or 3 reactive sub-units of the conjugate of formula I with one another that a conjugate having covalent bonds is formed, and isolating the conjugate.

10. A process according to claim 9 which comprises:

- a) for the preparation of a conjugate of formula I in which the sub-units are linked by an amide bond, condensing a sub-unit containing an amino group with the complementary sub-unit containing a carboxylic acid group or with a reactive derivative thereof, or
- b) for the preparation of a conjugate of formula I in which the sub-units are linked by a disulfide bond, treating two complementary sub-units that both contain a mercapto group with a mild oxidizing reagent, or
- c) for the preparation of a conjugate of formula I in which the sub-units are linked by a disulfide bond, substituting a reactive substitutable functional derivative of the mercapto group of one sub-unit with the complementary sub-unit containing a mercapto group, or
- d) for the preparation of a conjugate of formula I in which the sub-units are linked by a carbon-sulfur bond, adding a sub-unit containing a mercapto group to the complementary sub-unit containing a carbon-carbon double bond or containing an epoxide function, or substituting a carbon-halogen bond by the mercapto group, or
- e) for the preparation of a conjugate of formula I in which the sub-units are linked by a carbon-nitrogen single or double bond, condensing an aldehyde group of one sub-unit with the complementary sub-unit containing an amino or hydrazino group, and, if desired, reducing the resulting carbon-nitrogen double bond to a carbon-nitrogen single bond with a reducing agent, or
- f) for the preparation of a conjugate of formula I in which the sub-units are linked by a carbon-nitrogen single bond, adding a sub-unit containing an amino or a hydrazino group to the complementary sub-unit containing an epoxide function, or substituting a carbon-halogen bond by the amino or hydrazino group.

11. Pharmaceutical preparations containing conjugates of cytokines with human immunoglobulin.

13. A method of treating viral infections and tumours in an animal including man, which comprises administering an effective amount of conjugates of cytokines with human immunoglobulin according to claim 1.

Form 10

COMMONWEALTH OF AUSTRALIA
PATENTS ACT 1952-69

COMPLETE SPECIFICATION

(ORIGINAL)

Class

Int. Class

Application Number:
Lodged:

Complete Specification Lodged:
Accepted:
Published:

Priority:

Related Art:

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Complete Specification for the invention entitled:

CONJUGATES OF CYTOKINES WITH IMMUNOGLOBULINS

The following statement is a full description of this invention, including the best method of performing it known to :
US

Conjugates of cytokines with immunoglobulins

The present invention relates to novel conjugates consisting of a cytokine and human immunoglobulin, if desired linked by means of an organic bridge-former, to processes for the preparation of such conjugates, to pharmaceutical preparations, and to the use of those conjugates for the treatment of viral infections and tumours.

Background of the invention

Cytokines are soluble proteins and glycoproteins that are secreted by somatic cells, especially immunocompetent cells, in response to a stimulation. Cytokines have a large number of biological activities. They are messenger molecules that take part in the regulation of the metabolic status of adjacent cells, are produced locally in the cells and are also normally effective only locally. Cytokines enhance or suppress the activity of an adjacent cell in respect of a specific property.

Examples of cytokines are mediators that influence monocytes and macrophages, such as macrophage migration inhibition factor (MIF), macrophage activating factor (MAF) or chemotactic factor for macrophages, mediators that influence polymorphonuclear leucocytes, such as leucocyte inhibition factor, leucocyte migration inhibition factor (LIF) or leucocyte migration promotor factor, mediators that influence lymphocytes, antigen-specific or non-specific helper cell factors or antibody production suppressor factors, mediators that act on other cells, such as lymphotoxins, tumour necrosis factor (TNF), colony stimulating factor (CSF), growth inhibition and growth promotor factors, especially B-cell growth factor (BCGF), insulin-like growth factor (IGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF) and others, and also the group of interferons (IFN) and the group of interleukins (IL).

Like most of the cytokines, interferons have a molecular weight in the range of from 10,000 to 40,000 Daltons. They protect cells against a large number of viruses, inhibit cell growth in culture, influence immune responses and increase the amount or activity of certain specific intracellular enzymes. Interferons are subdivided into three classes, namely interferon α , β and γ . Natural human interferon alpha (huIFN α) is produced by leucocytes and lymphoblastoid cells and comprises at least a dozen different polypeptides which differ from one another in their amino acid composition and in their degree of glycosylation. Natural human interferon beta (huIFN β) is formed by practically all body cells, for example by fibroblasts and to a slight extent also by lymphoblastoid cells. Natural human interferon gamma (huIFN γ), also called immune interferon, is produced by T-lymphocytes or NK cells. In contrast to IFN α and IFN β , IFN γ is acid-labile (pH 2) and is distinctly different in its serological properties.

Human cytokines for therapeutic use can be obtained by culturing stimulated normal human cells. This source is of little practical importance, however, owing to the limited availability of cultivatable human cells and the inherent separation problems and costs. Certain cytokines can also be obtained by culturing continuous human cell lines. The method best suited to the industrial preparation of cytokines, however, is the cultivation of gene-technologically modified bacteria, fungi or mammalian cell lines in which the genetic information for the synthesis of the desired cytokine has been incorporated by recombinant gene technology. This method also renders possible the preparation of compounds that differ from natural compounds and consequently have different and better properties for certain intended uses.

The therapeutic use of cytokines, for example of interleukin 2, interferon α or interferon γ , is not without problems. They are normally quickly broken down and/or excreted. In the case of systemic administration it is difficult to build up and maintain the high plasma concentration necessary for an antiviral or anti-tumour activity. On the other hand, undesired side effects arise, which are possibly attributable to the passage of the cytokines through the blood-brain barrier. There is

therefore a pressing need to find ways and means on the one hand to achieve therapeutically effective high concentrations of cytokine in the plasma, and on the other hand to limit or reduce undesired side effects.

Object of the invention

The object of the present invention is to render cytokines available in a form that makes it possible to increase their residence time and concentration in blood plasma and tissue. An especially suitable solution to this problem is provided by conjugates consisting of a cytokine and human immunoglobulin, if desired linked by means of an organic bridge-former, that surprisingly have distinctly longer biological half-lives than free cytokines. Also described are a process for the preparation of the conjugates, pharmaceutical preparations and their use in the treatment of viral infections and tumours.

Description of the invention

The invention relates to conjugates of cytokines with human immunoglobulin.

A cytokine is a soluble protein or glycoprotein, as synthesised and if need be secreted, in response to a stimulation, by somatic cells, for example immunocompetent cells, in order to effect a metabolic change in adjacent cells, for example in order to influence their growth properties, and is also a related compound that differs from a natural cytokine as a result of the replacement, omission or addition of one or more amino acids and/or carbohydrate residues, or is a fragment of such a compound that retains the biological activity. For example, one, two, three or four amino acids of a natural peptide may be replaced by other amino acids or may be omitted completely, one, two, three or four additional amino acids may be present, for example at the amino end of the peptide, one or two carbohydrate residues of the natural glycoprotein may be replaced by other carbohydrate residues or one, two, three or all carbohydrate residues may be missing. Furthermore, related compounds may be composed, for example, also of parts of two different cytokines.

Examples of cytokines of this definition are the naturally occurring factors, such as macrophage migration inhibition factor (MIF), macrophage activating factor (MAF), interleukin 1, 2, 3, 4, 5 or 6, colony stimulating factor (CSF), lymphotoxin, B-cell growth factor (BCGF), interferon alpha (IFN α), interferon beta (IFN β) or interferon gamma (IFN γ), especially the corresponding human cytokines, also compounds that are related or identical to the natural factors and are produced by recombinant gene technology in prokaryotes, in eukaryotes or mammalian cells, such as recombinant MAF, MIF, interleukin, CSF, BCGF, lymphotoxin, IFN α , for example IFN α_1 , IFN α_2 , IFN α , etc. up to IFN α_{14} , and IFN α /A, IFN α /B, IFN α /D, IFN α /F or IFN α /B-D hybrids, IFN β or IFN γ , and also fragments of the natural or recombinant compounds that retain the biological activity. Hormones and antibodies (also called immunoglobulins) that are secreted by B-lymphocytes after stimulation by antigens and/or mitogens do not fall within the definition of cytokines.

An immunoglobulin is a human immunoglobulin (antibody) that belongs to the A, D, E, G or M class of immunoglobulins, preferably immunoglobulin G or M. The variable region of the immunoglobulin has an arbitrary specificity. Preferably, the immunoglobulin in the conjugate of the invention is a mixture of antibodies of the same class of immunoglobulin but of as many different specificities as possible, such as can be obtained from the blood of healthy humans. An immunoglobulin in the conjugate of the invention can, however, also be a polyclonal or monoclonal human antibody specific to an antigen that is foreign to the body.

A conjugate is a hybrid molecule in which one or more cytokines and one or more immunoglobulins are combined. It is a chemical complex in which the coupling is effected by a chemical bond, for instance an ionic or covalent bond, or optionally by a bridge-former, for example an organic bridge-former, or an association of the molecules involved. The mentioned chemical complexes or associations are preferably stable under physiological conditions.

The invention relates preferably to conjugates of the formula



in which Ck is a residue of a natural human cytokine or of a recombinant cytokine, Ig is a residue of a human immunoglobulin and Z is a covalent bond or a covalently bonded organic bridge-former, and in which Ck, Ig and/or Z also may occur several times.

The residue Ck is mono- or poly-valent, for example di- or tri-valent, depending on the number of hydrogen atoms and/or hydroxy groups that are replaced by bonds to bridge-formers Z or to immunoglobulin residues Ig.

The residue Ig is mono- or poly-valent, for example di-, tri-, tetra-, penta- or hexa-valent, depending on the number of hydrogen atoms and/or hydroxy groups that are replaced by bonds to bridge-formers Z or to cytokine residues Ck.

In conjugates of the formula I in which Z is a covalent bond, cytokines and immunoglobulins may be bonded by an amide bond between a terminal amino function or a side-chain amino function of the one peptide and a terminal carboxylic acid function or a side-chain carboxylic acid function of the other peptide, or by a carbon-nitrogen bond between a terminal amino function or a side-chain amino function of the one peptide and a carbohydrate residue of the other glycoprotein.

In such conjugates in which Z is a covalent bond, the residues of the cytokine and of the immunoglobulin are bonded preferably by an amide bond between the terminal carboxy function of the cytokine and an amino function of the immunoglobulin, or by a carbon-nitrogen bond between an amino function of the cytokine and a carbohydrate residue of the immunoglobulin.

An organic bridge-former Z is a polyvalent, for example di-, tri- or tetra-valent, hydrocarbon radical that is if desired substituted and/or in which one or more carbon atoms are if desired replaced by hetero

atoms, and that is bonded covalently to the cytokine and to the immunoglobulin residue by an amide, ester, amine, thioether, thioester or disulfide single bond or by a carbon-nitrogen double bond. Such hydrocarbon radicals have up to 30, especially from 4 to 20, carbon atoms and are, for example, of aliphatic, cycloaliphatic, aromatic, araliphatic or aliphatic-cycloaliphatic nature. Substituents of such hydrocarbon radicals 2 are, for example, one or more, for example two, if desired protected hydroxy groups, oxo groups, if desired protected amino groups, imino, nitro, lower alkoxy, lower alkylamino, di-lower alkylamino or lower alkyl groups, if desired protected hydroxy-lower alkyl groups, if desired protected amino-lower alkyl groups, if desired protected mercapto-lower alkyl groups, methylthio-lower alkyl groups, if desired protected carboxy-lower alkyl groups, carbamoyl-lower alkyl, amidino-lower alkyl, aryl-lower alkyl, for example phenyl-lower alkyl or if desired protected hydroxyphenyl-lower alkyl groups, and heteroaryl-lower alkyl groups, for example if desired protected indolyl-lower alkyl or imidazolyl-lower alkyl groups. In such hydrocarbon radicals 2 hetero atoms that replace carbon atoms are, for example, oxygen, sulfur and/or nitrogen, the nitrogen atoms if desired being substituted by lower alkyl or being part of a cycloaliphatic ring.

Divalent hydrocarbon radicals are, for example, lower alkylene, cycloalkylene, phenylene or combinations of these radicals, such as lower alkylene-cycloalkylene, lower alkylene-phenylene, lower alkylene-cycloalkylene-lower alkylene, lower alkylene-phenylene-lower alkylene or the like. Divalent hydrocarbon radicals in which hetero atoms replace carbon atoms are, for example, amino-lower alkylene, lower alkylene-amino-lower alkylene, hydrazino-phenylene-amino, oxy-lower alkylene, lower alkylene-oxy-lower alkylene, thio-lower alkylene, lower alkylene-thio-lower alkylene, lower alkylene-dithio-lower alkylene and azacycloalkylene in which a nitrogen atom replaces a ring carbon atom, and combinations of the mentioned radicals, such as, for example, from 2 to 10 repeated units of the same or different amino-lower alkylene radicals that if desired are bonded to hydrazino-phenylene, amino-lower alkylene-dithio-lower alkylene-amino, lower alkylene-azacycloalkylene, lower alkylene-azacycloalkylene-thio-lower alkylene, lower alkylene-phenylene-azacycloalkylene,

lower alkylene-cycloalkylene-azacycloalkylene, lower alkylene-oxy- or amino-lower alkylene-azacycloalkylene, azacycloalkylene-phenylene-azacycloalkylene, lower alkylene-amino-phenylene-amino-lower alkylene, lower alkylene-oxy-lower alkylene-oxy-lower alkylene or the like.

Trivalent hydrocarbon radicals are, for example, lower alkylene-methylidene, phenylene-lower alkylidene and similar radicals and the corresponding radicals in which nitrogen atoms replace carbon atoms are, for example, amino-phenylene-hydrazinyldene. Tetravalent hydrocarbon radicals are, for example, lower alkanediylidene, cycloalkanediylidene or similar radicals.

The term "lower" used in lower alkyl, lower alkoxy, lower alkylene or the like indicates such groups having from 1 to 7, preferably from 1 to 4, carbon atoms. Lower alkyl preferably has up to 4 carbon atoms and is, for example, methyl, ethyl, propyl, isopropyl, butyl, isobutyl, sec.-butyl or tert.-butyl. Lower alkoxy preferably has up to 4 carbon atoms and is, for example, methoxy, ethoxy, propoxy or butoxy. Lower alkylene preferably has up to 7 carbon atoms and is, for example, methylene, ethylene, 1,3-propylene, 1,4-butylene or 1,6-hexylene.

Cycloalkylene preferably has from 3 to 8 carbon atoms and is, for example, 1,2-cyclopentylene, 1,3-cyclopentylene, 1,4-cyclohexylene or 1,5-cyclooctylene. Phenylene is 1,2-, 1,3- or 1,4-phenylene. Azacycloalkylene is, for example, 1-aza-1,2-cyclopentylene, 1-aza-1,3-cyclopentylene or 1-aza-1,3-cyclohexylene.

Protecting groups are those customarily used in peptide and/or carbohydrate chemistry.

A protected amino group is, for example, in the form of a readily cleavable acylamino, arylmethylamino, 2-acyl-lower alkylenyl-1-amino or silylamino group or in the form of an azido group. Preferred amino-protecting groups are acyl radicals of carbonic acid semiesters, especially tert.-butoxycarbonyl, if desired substituted benzyloxycarbonyl, diphenylmethoxycarbonyl, 2-halo-lower alkoxycarbonyl, trityl or formyl.

A hydroxy group is protected, for example, in the form of a readily cleavable acyloxy group, especially an oxycarbonyloxy group, such as tert.-butoxycarbonyloxy, benzyloxycarbonyloxy or 2-halo-lower alkoxy-carbonyloxy, or a readily cleavable ether group, especially arylmethoxy, such as trityloxy, 1-oxyalkoxy, such as 1-ethoxyethoxy, or 2-tetrahydro-pyranyloxy, and silyloxy, such as tert.-butyldimethylsilyloxy.

A carboxy group is protected, for example, in the form of a readily cleavable ester group. Preferred carboxy-protecting groups are tert.-butoxy, if desired substituted benzyloxy, diphenylmethoxy, allyloxy and 2-(trimethylsilyl)ethoxy.

A mercapto group is protected, for example, in the form of a readily cleavable thioester, monothioacetal, thioether or disulfide group.

A bridge-former Z is linked with the cytokine residue and the immunoglobulin residue, for example by an amide bond between the terminal amino function or a side-chain amino function of the cytokine or immunoglobulin and a carboxy function of Z, by an amide bond between a terminal carboxy function or a side-chain carboxy function of the cytokine or immunoglobulin and an amino function of Z, by a nitrogen-carbon single or double bond between a terminal or a side-chain amino function of the cytokine or immunoglobulin and a carbon atom of Z, by a carbon-nitrogen single or double bond between a carbohydrate residue of the immunoglobulin and an amine or hydrazine function of Z, by an ester bond between a hydroxy function of an amino acid or of a carbohydrate residue of the cytokine or immunoglobulin and a carbon atom of Z, by a thioether or thioester bond between a mercapto function of a cysteine residue of the cytokine or of the immunoglobulin and a carbon atom of Z or by a disulfide bridge between a mercapto function of a cysteine residue of the cytokine or immunoglobulin and a mercapto function of Z.

A bridge-former Z is, for example, 1-oxo-lower alkylene or 1-oxo-lower alkylene-amino, such as carbonyl-propylene, carbonyl-propylene-amino, carbonyl-ethylene, carbonyl-ethylene-amino, or preferably carbonyl-

methylene. Preferably, such a group Z is linked to the cytokine or immunoglobulin at the carbonyl group and at the amine radical by an amide bond and, if desired, is bonded at the lower alkylene end by a carbon-nitrogen single bond.

Another bridge-former Z is, for example, a divalent or trivalent radical consisting of from 1 to approximately 10, preferably from 1 to 3, carbonyl-methylene-amino groups and, if desired, a carbonyl-methylene, a phenylene-hydrazino or a phenylene-hydrazinylidene group. In such a carbonyl-methylene-amino group, if desired methylene is substituted by lower alkyl, hydroxy-lower alkyl, amino-lower alkyl, mercapto-lower alkyl, methylthio-lower alkyl, carboxy-lower alkyl, carbamoyl-lower alkyl, amidino-lower alkyl, phenyl-lower alkyl, hydroxyphenyl-lower alkyl, indolyl-lower alkyl or imidazolyl-lower alkyl, for example as in the residues of the naturally occurring amino acids glycine, alanine, valine, leucine, isoleucine, serine, threonine, lysine, cysteine, methionine, asparagine, glutamine, aspartic acid, glutamic acid, arginine, phenylalanine, tyrosine, tryptophan and histidine. Methylene and amino in a carbonyl-methylene-amino group may also be bridged by lower alkylene, for example as in the residue of the naturally occurring amino acid proline. The carbonyl-methylene-amino groups are linked to one another and to the carbonyl-methylene group by amide bonds. Preferably, such a bridge-former is linked with the cytokine or immunoglobulin at the carbonyl end and at the amino end by an amide bond and, if applicable, at the methylene end or at the hydrazine end by a carbon-nitrogen single bond and at the hydrazinylidene end by a carbon-nitrogen double bond.

Examples of such bridge-formers are the tripeptide residues glycine-alanine-arginine, isoleucine-alanine-tyrosine, the pentapeptide residues leucine-glycine-alanine-alanine-arginine, glycine-glycine-leucine-alanine-tyrosine, alanine-isoleucine-alanine-isoleucine-lysine and bridge-formers in which those peptide residues are bonded at the nitrogen end to carbonyl-methylene, 4-phenylene-hydrazino or 4-phenylene-hydrazinylidene.

Another bridge-former 2 is a divalent radical amino-lower alkylene-thio, preferably amino-ethylene-thio, oxo-lower alkylene-thio, preferably carbonyl-methylene-thio or carbonyl-ethylene-thio, methylene-carbonyl-methylene-thio or 1-imino-lower alkylene-thio, preferably carbonimidoyl-propylene-thio. In such a bridge-former the thio function is bonded to a cysteine residue of the cytokine or immunoglobulin by a disulfide bridge.

Another bridge-former 2 is the divalent radical amino-lower alkylene-dithio-lower alkylene-amino, preferably amino-ethylene-dithio-ethylene-amino, or oxo-lower alkylene-dithio-oxo-lower alkylene, preferably carbonyl-methylene-dithio-methylene-carbonyl, carbonyl-ethylene-dithio-ethylene-carbonyl or carbonyl-propylene-dithio-propylene-carbonyl, oxo-lower alkylene-dithio-imino-lower alkylene, for example carbonyl-methylene-, -ethylene- or -propylene-dithio-propylene-carbonimidoyl, or imino-lower alkylene-dithio-imino-lower alkylene, for example carbonimidoyl-propylene-dithio-propylene-carbonimidoyl or carbonimidoyl-butylene-dithio-butylene-carbonimidoyl. In such bridge-formers the carbonyl function is linked to the cytokine or immunoglobulin preferably by an amide bond and the carbonimidoyl function preferably by a thio-iminoester bond.

Another bridge-former 2 is, for example, a divalent radical that is formed on the one hand from oxo-lower alkylene, such as carbonyl-ethylene, from carbonyl-cycloalkylidene, such as carbonyl-1,4-cyclohexylene, from methylene-carbonyl-methylene, methylene-carboxy-lower alkylene, such as methylene-carboxy-methylene, from methylene-carbox-amido-lower alkylene, such as methylene-carboxamido-methylene, or from carbonyl-phenylene, preferably carbonyl-1,3-phenylene, and on the other hand from 1-succinimidylene-3, 1-succinimidylene-3-thio-lower alkylene-amino, such as 1-succinimidylene-3-thio-ethylene-amino, from 1-succinimidylene-3-thio-oxo-lower alkylene in which lower alkylene is, for example, ethylene or, preferably, propylene, from 1-succinimidylene-3-thio-methylene-carbonyl-methylene or from 1-succinimidylene-3-thio-ethoxymethylene-carbonyl-methylene or from 1-succinimidylene-3-thio-imino-lower alkylene in which lower alkylene is preferably butylene. The nitrogen atom of succinimidylene. A 1-succinimidylene-3 radical of a

bridge-former is bonded, for example, at carbon 3 to a mercapto group of a cysteine residue of the cytokine or immunoglobulin. Especially preferred are the bridge-formers carbonyl-1,3-phenylene-1-succinimidylene-3-thio-ethylene-carbonyl and carbonyl-1,3-phenylene-1-succinimidylene-3-thio-propylene-carbonimidoyl, which are linked to the cytokine and the immunoglobulin by amide bonds and a thioiminoester bond.

Another bridge-former Z is, for example, a divalent lower alkylene radical, a trivalent lower alkylene-methylidene radical or a tetravalent lower alkanediylidene radical, it being possible for these radicals to be substituted by lower alkyl, for example 1,5-pentylene, 3-methyl-1,5-pentylene, 3,3-dimethyl-1,5-pentylene, 1,6-hexylene, 1,4-butylenemethylidene, 1,5-pentdiylidene, 3-methyl-1,5-pentdiylidene, or 3,3-dimethyl-1,5-pentdiylidene, preferably 1,5-pentylene and 1,5-pentdiylidene. Such a bridge-former is preferably linked with the cytokine and the immunoglobulin by a carbon-nitrogen single bond or double bond.

Another bridge-former Z is, for example, a divalent lower alkylene radical having 2 hydroxy groups, for example 2,3-dihydroxy-1,4-butyleneor, preferably, 2,4-dihydroxy-1,5-pentylene, or lower alkylene-oxy-lower alkylene and lower alkylene-oxy-lower alkylene-oxy-lower alkylene having 2 hydroxy groups, for example (2-hydroxy-1,3-propylene)-oxy-(2-hydroxy-1,3-propylene) or (2-hydroxy-1,3-propylene)-oxy-(1,4-butylen)-oxy-(2-hydroxy-1,3-propylene). Such a bridge-former is preferably linked to the cytokine and immunoglobulin by a carbon-nitrogen single bond.

Another bridge-former Z is, for example, a divalent carbonyl-amino-phenylene-amino-carbonyl radical, which if desired is substituted by lower alkyl groups, for example carbonyl-amino-1,4-phenylene-amino-carbonyl or carbonyl-amino-1-methyl-2,4- or -2,6-phenylene-amino-carbonyl, or the corresponding thiocarbonyl radicals. Such a bridge-former is linked to an amino group of the cytokine and of the immunoglobulin by an amide bond and is thus a urea or thiourea derivative.

Compared with cytokines alone, the conjugates of the invention surprisingly have a substantially higher residence time half life in blood plasma and tissue. This observation can be made, for example, by injecting experimental animals, for example mice, rats or rabbits, intravenously, subcutaneously or intramuscularly with cytokines only, with conjugates according to the invention, and with mixtures of non-conjugated cytokines and immunoglobulin, taking blood and tissue samples at various intervals and ascertaining their content of cytokine or cytokine conjugates. This test demonstrates that, compared with cytokines alone or cytokine-immunoglobulin mixtures, the residence time half life of the conjugates of the invention is higher by a factor of 5 or more.

The conjugates of the invention can therefore be used in the same manner as the corresponding cytokines on their own for the treatment of viral infections and/or tumours. Compared with the cytokines themselves, however, the conjugates of the invention have the advantage that they can be used in distinctly smaller amounts owing to their longer residence time in the organism and especially in the bloodstream and tissue. Furthermore, it is to be expected that the conjugates of the invention, when applied directly to the site at which they are to act, will remain there longer and thus bring about fewer side effects, for example, in the central nervous system.

The invention relates especially to conjugates of the formula I in which

Ck is a residue of a natural or recombinant human interferon, of interleukin, of a related compound or of a fragment that retains the biological activity, for example interferon alpha, beta or gamma, interleukin 2 or a recombinant protein or glycoprotein in which one, two, three or four amino acids of an interferon or interleukin have been replaced or omitted, in addition one, two, three or all of the carbohydrate residues of an interferon or interleukin are missing, parts of two different interferons or interleukins are combined with one another, or only the biologically active part of the interferon or interleukin is present,

Ig is a residue of a natural human immunoglobulin, for example an immunoglobulin G or M from the blood of a healthy human,

Z is a covalent bond or a covalently bonded di-, tri- or tetra-valent hydrocarbon radical which, if desired, is substituted and/or in which, if desired, one or more, for example two, three or four, carbon atoms have been replaced by oxygen, sulfur and/or unsubstituted or substituted nitrogen atoms, for example lower alkylene, cycloalkylene, phenylene and combinations of the three mentioned radicals with one another and/or with oxy, thio and unsubstituted or lower alkyl-substituted amino, also with azacycloalkylene, it being possible for the said radicals to be substituted by hydroxy, oxo, amino, imino, lower alkoxy, lower alkylamino, di-lower alkylamino, lower alkyl, hydroxy-lower alkyl, amino-lower alkyl, mercapto-lower alkyl, methylthio-lower alkyl, carboxy-lower alkyl, carbamoyl-lower alkyl, amidino-lower alkyl, phenyl-lower alkyl, hydroxyphenyl-lower alkyl, indolyl-lower alkyl or by imidazolyl-lower alkyl, also lower alkylene-methylidene, phenylene-lower alkylidene, amino-phenylene-hydrazinylidene, lower alkanediylidene or cycloalkanediylidene,

and in which Ck, Ig and/or Z also may occur several times, for example twice or three times.

The invention relates more especially to conjugates of the formula I in which

Ck is a residue of a natural or recombinant human interferon or of a related compound, for example natural interferon alpha, natural interferon gamma, recombinant interferon alpha, for example IFN α_1 , IFN α_2 , IFN α_3 to IFN α_{16} , and IFN α/A , IFN α/B , IFN α/D , IFN α/F or IFN $\alpha/B-D$ hybrid in glycosylated or non-glycosylated form and with, if desired, replaced, missing, or one to four additional, amino acids, or recombinant interferon gamma,

Ig is a residue of a natural human immunoglobulin, for example an immunoglobulin G or M from the blood of a healthy human, and

Z is a covalent bond or a covalently bonded divalent hydrocarbon radical which, if desired, is substituted by oxo or imino and/or in which one or more, for example two, three or four, carbon atoms have been replaced by oxygen, sulfur and/or nitrogen atoms, for example unsubstituted or oxo- and/or imino-substituted lower alkylene and combinations of that radical with oxy, thio, amino, phenylene and/or azacycloalkylene, for example 1-oxo-lower alkylene, for example carbonyl-propylene, carbonyl-ethylene or carbonyl-methylene, 1-oxo-lower alkylene-amino, for example carbonyl-propylene-amino or carbonyl-ethylene-amino, amino-lower alkylene-thio, for example amino-ethylene-thio, oxo-lower alkylene-thio, for example carbonyl-methylene-thio, carbonyl-ethylene-thio or methylene-carbonyl-methylene-thio, 1-imino-lower alkylene-thio, for example carbonimidoyl-propylene-thio, amino-lower alkylene-dithio-lower alkylene-amino, for example amino-ethylene-dithio-ethylene-amino, oxo-lower alkylene-dithio-oxo-lower alkylene, for example carbonyl-methylene-dithio-methylene-carbonyl, carbonyl-ethylene-dithio-ethylene-carbonyl or carbonyl-propylene-dithio-propylene-carbonyl, oxo-lower alkylene-dithio-imino-lower alkylene, for example carbonyl-ethylene-dithio-propylene-carbonimidoyl, imino-lower alkylene-dithio-imino-lower alkylene, for example carbonimidoyl-propylene-dithio-propylene-carbonimidoyl, carbonyl-phenylene-succinimidylene, for example carbonyl-1,3-phenylene-1-succinimidylene-3, carbonyl-phenylene-succinimidylene-thio-lower alkylene-amino, for example carbonyl-1,3-phenylene-1-succinimidylene-3-thio-ethylene-amino, carbonyl-phenylene-succinimidylene-thio-oxo-lower alkylene, for example carbonyl-1,3-phenylene-1-succinimidylene-3-thio-ethylene-carbonyl, or carbonyl-phenylene-succinimidylene-thio-imino-lower alkylene, for example carbonyl-1,3-phenylene-1-succinimidylene-3-thio-propylene-carbonimidoyl.

The invention relates more especially to conjugates of the formula I in which Ck is a residue of a natural or recombinant human interferon alpha, for example IFN α_{2b} or IFN α /B-D hybrid, Ig is a residue of a natural human immunoglobulin G, for example as can be obtained from the blood of a healthy human, and Z is a covalently bonded oxo- and/or imino-substituted lower alkylene-dithio-lower alkylene radical, for example carbonyl-ethylene-dithio-ethylene-carbonyl, carbonyl-ethylene-dithio-propylene-carbonimidoyl or carbonimidoyl-propylene-dithio-propylene-carbonimidoyl.

Most especially the invention relates to the conjugates mentioned in the Examples.

The invention relates also to a process for the preparation of cytokines with human immunoglobulin which comprises reacting a cytokine with human immunoglobulin and, if desired, a bridge-former. The conjugation is effected according to methods that are known per se.

The invention relates preferably to a process for the preparation of conjugates of the formula

Ck-Z-Ig

(I),

which comprises so reacting 2 or 3 reactive sub-units of the conjugate of formula I with one another that a conjugate having covalent bonds is formed, and isolating the conjugate.

The present invention relates especially to the said process which comprises:

a) for the preparation of a conjugate of formula I in which the sub-units are linked by an amide bond, condensing a sub-unit containing an amino group with the complementary sub-unit containing a carboxylic acid group or with a reactive derivative thereof, or

b) for the preparation of a conjugate of formula I in which the sub-units are linked by a disulfide bond, treating two complementary sub-units that both contain a mercapto group with a mild oxidizing reagent, or

c) for the preparation of a conjugate of formula I in which the sub-units are linked by a disulfide bond, substituting a reactive substitutable functional derivative of the mercapto group of one sub-unit with the complementary sub-unit containing a mercapto group, or

d) for the preparation of a conjugate of formula I in which the sub-units are linked by a carbon-sulfur bond, adding a sub-unit containing a mercapto group to the complementary sub-unit containing a carbon-carbon double bond or containing an epoxide function, or substituting a carbon-halogen bond by the mercapto group, or

e) for the preparation of a conjugate of formula I in which the sub-units are linked by a carbon-nitrogen single or double bond, condensing an aldehyde group of one sub-unit with the complementary sub-unit containing an amino or hydrazino group, and, if desired, reducing the resulting carbon-nitrogen double bond to a carbon-nitrogen single bond with a reducing agent, or

f) for the preparation of a conjugate of formula I in which the sub-units are linked by a carbon-nitrogen single bond, adding a sub-unit containing an amino or a hydrazino group to the complementary sub-unit containing an epoxide function, or substituting a carbon-halogen bond by the amino or hydrazino group.

Pairs of sub-units that can be linked together according to process a) by an amide bond are, for example, a cytokine and an immunoglobulin that each contain a free carboxy group or a free amino group, a cytokine and an immunoglobulin substituted by one or more bridge-formers containing a free carboxy and/or amino group, a cytokine substituted by one or more bridge-formers containing a free carboxy and/or amino group and an immunoglobulin, or a cytokine and an immunoglobulin that each carry a bridge-former moiety containing a free carboxy group and a free amino group, respectively, and these two moieties together form a bridge-former 2. Such pairs of sub-units or mixtures of three sub-units consisting of cytokine, immunoglobulin and a bridge-former containing a free carboxy and a free amino function are treated with a condensing agent. Suitable condensing agents are those that are conventionally used in peptide chemistry, for example carbodiimides, for example N,N'-dicyclohexylcarbodiimide, preferably water-soluble carbodiimides such as N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide or N-ethyl-N'-(3-N-morpholinopropyl)-carbodiimide, or isocyanides, for example 2-(N-morpholino-

ethyl)-isocyanide, if desired in the presence of a tertiary amine, for example triethylamine, N-methylmorpholine or 4-dimethylaminopyridine, and of an activated ester-forming hydroxy compound, for example N-hydroxy-succinimide, N-hydroxy-5-norbornene-2,3-dicarboxylic acid imide or 1-hydroxybenzotriazole. Other suitable condensing agents are, for example, 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline or activated phosphates, such as diphenylphosphoroazidate or diethylphosphorocyanide, if desired in the presence of a tertiary amine. The condensation is carried out in an aqueous solvent that may contain an organic or inorganic buffer and/or a miscible organic diluent, for example a lower alkanol, for example tert.-butanol, a lower alkanediol, for example glycol, dimethylformamide, acetonitrile or the like, at temperatures of from approximately -20°C to approximately +50°C, preferably from 5°C to room temperature.

Reactive functional derivatives of sub-units in process a) can be formed in situ and are, for example, reactive carboxylic acid derivatives, for example activated carboxylic acid esters, reactive mixed anhydrides, reactive cyclic amides, isocyanates or isothiocyanates.

Activated esters of carboxylic acids are, for example, of the vinyl ester type, for example actual vinyl esters, carbamoyl vinyl esters or 1-lower alkylvinyl esters, esters of the amidine type, for example N,N'-disubstituted amidino esters that can be obtained by treating the corresponding acid with a suitable N,N'-disubstituted carbodiimide, for example with N,N'-dicyclohexylcarbodiimide, or also N,N-disubstituted amidino esters, suitable aryl esters, especially phenyl esters substituted by electron-attracting substituents, for example 4-nitrophenyl, 2,4,5-trichlorophenyl, pentachlorophenyl or 4-phenyldiazophenyl ester, cyanomethyl esters, thioesters, especially nitrophenyl thioester, or preferably amino or amido esters that have been obtained, for example, by treating the corresponding acid with an N-hydroxyamino or N-hydroxyamido compound, for example with N-hydroxysuccinimide, N-hydroxypiperidine, N-hydroxyphthalimide, N-hydroxy-5-norbornene-2,3-dicarboxylic acid imide or 1-hydroxybenzotriazole.

Reactive mixed anhydrides of acids are, for example, anhydrides with inorganic acids, for example acid halides, especially acid chlorides, anhydrides with carbonic acid lower alkyl semiesters, anhydrides with dihalogenated, especially dichlorinated, phosphoric acids or phosphoric acid derivatives, anhydrides with strong carboxylic acids, for example trifluoroacetic acid, or anhydrides with organic sulfonic acids, for example lower alkanesulfonic acids or arylsulfonic acids, such as methane- or p-toluene-sulfonic acid.

Suitable cyclic amides are especially those with five-membered aromatic diazaheterocycles, for example amides with imidazole or pyrazole, for example with 3,5-dimethylpyrazole.

Reactive carboxylic acid derivatives are reacted with a reactant containing a free amino group under the same reaction conditions as those described above for non-activated sub-units except that the condensing agent can be omitted. Preferably, an acid-binding compound is added, for example an alkali metal carbonate or hydrogen carbonate, for example sodium or potassium carbonate or hydrogen carbonate, customarily together with the corresponding sulfate, or an organic base, for example a sterically hindered tri-lower alkylamine, for example ethyldiisopropylamine.

Preferably, the reactive carboxylic acids are derivatives of the bridge-former, for example dicarboxylic acid dichlorides, activated dicarboxylic acid diesters, diisocyanates, for example phenylene-1,4-diisocyanate or 1-methyl-phenylene-2,4-diisocyanate, or diisothiocyanates.

The starting materials for process a) are known or can be prepared according to known processes, for example by a process as described hereinbefore or hereinafter.

Pairs of sub-units that can produce a disulfide bond in accordance with process b) or c) are, for example, a cytokine and an immunoglobulin that both contain a bridge-former moiety containing a mercapto function and in

which the bridge-former moieties together form the radical 2. In process c) the mercapto group of one of the two sub-units is in the form of a reactive substitutable functional derivative.

Suitable oxidizing agents for process b) of the present invention are especially mild oxidizing agents, for example iodine, 1,2-diiodoethylene, o-iodobenzoic acid, 1,10-phenanthroline and copper sulfate, potassium hexacyanoferrate(III) or, preferably, oxygen or air. The reaction is carried out in water or aqueous buffer solution at a pH of from approximately 3 to approximately 9, preferably close to the neutral point, and at temperatures of from approximately -5°C to approximately room temperature. The aqueous reaction medium contains, if desired, a miscible organic solvent, for example a lower alcohol, for example tert.-butanol, dimethylformamide, acetonitrile or the like, and/or an alkali metal iodide, for example sodium or potassium iodide.

A reactive substitutable functional derivative of the mercapto group of a sub-unit in process c) can be formed in situ and is, for example, a sulfenyl halide, sulfenamide, sulfenylsulfone, thiosulfate or, preferably, a mixed disulfide.

A sulfenyl halide is, for example, a sulfenyl chloride or a sulfenyl bromide. A sulfenamide is derived, for example, from a cyclic amine, a dicarboxylic acid imide or a heterocycle and is, for example, N-sulfenylpiperidine, N-sulfenylsuccinimide, N-sulfenylphthalimide, N-sulfenyl-5-norbornene-2,3-dicarboxylic acid imide, 1-sulfenylbenzotriazole or 1-sulfenylimidazole.

A mixed disulfide is, for example, a disulfide with an aromatic thiol, for example with thiophenol substituted by halogen, nitro, cyano or carboxy, for example with o-chlorothiophenol, 2,4-dichlorothiophenol or p-nitrothiophenol, or a disulfide with a heteroaromatic thiol, for example with pyridinethiol, preferably with 2-pyridinethiol, with mercaptopyridinecarboxylic acid, quinolinethiol, 2-mercaptobenzimidazole, 2-mercaptobenzothiazole or with 2-mercaptobenzoxazole.

A sulphenylsulfone is, for example, a lower alkylthiosulfonate, for example a methanethiosulfonate, or a benzenethiosulfonate, which, if desired, is substituted by methyl, nitro or halogen, for example p-toluenethiosulfonate.

In a thiosulfate the organic radical containing the derivatised mercapto function is bonded to the sulfur. Preferably, the thiosulfate is in anionic form, for example an alkali metal thiosulfate, for example sodium or potassium thiosulfate.

There is especially preferred as the reactive substitutable functional derivative of the mercapto group in process c) a mixed disulfide, especially a disulfide with 2-pyridinethiol.

The reactive functional derivatives of the mercapto group are prepared according to known customary methods from compounds having a free mercapto function or a mercapto function derivatised in some other manner.

In process c) the reactant containing a free mercapto group and the reactant containing a reactive substitutable functional derivative of the mercapto group are reacted in water or, preferably, in an aqueous buffer, at a pH of from approximately 3 to approximately 9, preferably close to the neutral point, that is to say at a pH of from 6 to 8, at temperatures of from approximately -20°C to approximately +50°C, preferably from -5°C to room temperature. If desired, the aqueous reaction medium contains a miscible organic solvent, for example a lower alkanol, for example tert.-butanol, dimethylformamide, acetonitrile or the like.

The starting materials for processes b) and c) are known or can be prepared by processes that are known per se, for example by a process as described hereinbefore or hereinafter. A substituted cytokine and a substituted immunoglobulin in which the substituents carry a mercapto group are prepared, for example, from the cytokine and from the immunoglobulin, respectively, by reaction with a compound containing a protected mercapto group and an activated carboxylic acid function, for example an activated

ester as described hereinbefore under process a). The protecting group of the mercapto function is then removed by processes that are known per se. For example, a disulfide, as described hereinbefore as a reactive substitutable functional derivative of the mercapto group, can be used as a protecting group during a process analogous to process a), and afterwards cleaved under mild reducing conditions, for example with a thiol, for example 2-mercaptoethanol, 3-mercapto-1,2-propanediol, thioglycolic acid, thioglycolic acid methyl ester or 3-mercaptopropionic acid, or preferably with a dithiol, for example dithiothreitol or dithioerythritol, in an aqueous buffer solution at a pH of from approximately 3 to approximately 6, preferably from 4 to 5, at temperatures of from approximately -5°C to approximately room temperature.

A substituted cytokine and a substituted immunoglobulin in which the substituents carry a mercapto function can be prepared, for example, also from a cytokine and from an immunoglobulin, respectively, by reaction with an iminothiolactone, for example 2-iminothiolane, in an aqueous buffer solution at a pH of from approximately 5 to approximately 9, preferably close to the neutral point, that is about pH 8, at temperatures of from approximately -5°C to approximately room temperature.

Especially preferred is a process in which a cytokine or an immunoglobulin is condensed with N-succinimidyl-3-(2-pyridyldithio)-propionate in accordance with process a), at the same time the appropriate complementary immunoglobulin or cytokine is reacted with 2-iminothiolane in the manner described above, and the two sub-units are linked to form a dithio bond according to process c). Preferably the reaction steps are carried out without intermediates being isolated.

Pairs of sub-units that together can form a carbon-sulfur bond according to process d) are, for example, cytokines and immunoglobulins substituted by bridge-former moieties, in which one bridge-former moiety carries a carbon-carbon double bond, a carbon-halogen bond or an epoxide function and the other bridge-former moiety carries a mercapto group, and the two bridge-former moieties together form the radical Z.

The carbon-carbon double bond in process d) is preferably substituted at one or both ends by an electron-attracting group, for example by a carbonyl group of a ketone, of an ester or of an amide, for example as in vinyl ketones, acrylic esters, acrylamides, substituted 2-butene-1,4-diones, for example quinones, fumaric esters, fumaric amides, maleic esters or, preferably, maleimides.

The halogen atom of a carbon-halogen bond in process d) is, for example, chlorine, bromine or iodine. The carbon-halogen bond is preferably activated by a carbon-carbon or a carbon-oxygen double bond, for example as in allyl halides, halomethyl ketones, for example chloromethyl ketones, haloacetic acids and haloacetic acid derivatives, for example iodo-, bromo- or chloro-acetates and -acetamides, α -chloro-lower alkane-carboxylates and the like.

The epoxide function in process d) is preferably a primary epoxide function, that is to say a monosubstituted oxirane, and can be obtained, for example, in situ from a halohydrin by treatment with a base.

In process d), the reactants are reacted in an aqueous solvent that, if desired, contains an organic or inorganic buffer and/or a miscible organic solvent, for example a lower alkanol, a lower alkanediol, dimethylformamide, acetonitrile or the like, preferably in the presence of an acid-binding agent, for example an alkali metal carbonate or hydrogen carbonate, for example sodium or potassium carbonate or hydrogen carbonate, or an alkaline earth metal carbonate, for example magnesium carbonate or calcium carbonate, or an organic base, for example a sterically hindered tri-lower alkylamine, for example ethyldiisopropylamine, at temperatures of from approximately -20°C to approximately $+50^{\circ}\text{C}$, preferably from -5°C to room temperature.

The starting materials for process d) are known or can be prepared according to processes that are known per se, for example according to one of the processes described hereinbefore or hereinafter. In particu-

lar, substituted cytokines or substituted immunoglobulins containing mercapto groups are prepared in the manner described for the starting materials for processes b) and c).

Pairs of sub-units that together can form a carbon-nitrogen single or double bond according to process e) are, for example, a cytokine containing a free amino group, or substituted by one or more bridge-formers containing a free amino or hydrazine function, and a reactive derivative of an immunoglobulin containing an aldehyde function, a reactive derivative of a cytokine containing an aldehyde function and an immunoglobulin containing a free amino group or containing a bridge-former having a free amino or hydrazine function, or a substituted cytokine and a substituted immunoglobulin in which one substituent contains an aldehyde function and the other, complementary substituent, contains an amino or hydrazino function, and the two substituents are together a bridge-former 2.

A conjugate of the formula I is also formed in process e) from a mixture containing a cytokine, an immunoglobulin and a reactive derivative of the bridge-former containing two aldehyde functions, for example a lower alkane dialdehyde, for example glutaraldehyde.

The condensation according to process e) is carried out in an aqueous solvent which, if desired, contains an organic or inorganic buffer and/or a miscible organic solvent, for example a lower alkanol, for example tert.-butanol, a lower alkanediol, for example glycol, diethylene glycol, polyethylene glycol, dimethylformamide, acetonitrile or the like, at temperatures of from approximately -20°C to approximately +50°C, preferably from -5°C to room temperature.

The conjugates obtainable in this manner containing carbon-nitrogen double bonds, that is to say imines or hydrazones, are, if desired, treated with a selective reducing agent that reduces the carbon-nitrogen double bond to the more stable carbon-nitrogen single bond, without affecting the amide bonds of the proteins. Such reducing agents are, for example, borohydrides, for example alkali metal borohydrides, for example sodium or potassium borohydride, or zinc borohydride, in which, if

desired, one or two hydrides have been replaced by lower alkoxy groups, for example as in sodium dimethoxyborohydride or sodium tert.-butoxyborohydride, or preferably in which one hydride has been replaced by a cyano group, as in sodium cyanoborohydride.

The reduction is carried out in an aqueous buffer solution at a pH of from approximately 3 to approximately 9, preferably of from 3 to 6 for reductions with sodium cyanoborohydride, and close to the neutral point, that is to say at a pH of from 6 to 8, for reductions with metal borohydrides that are unsubstituted or substituted by lower alkoxy groups, at temperatures of from approximately -20°C to approximately +50°C, preferably from -5°C to room temperature. If desired, the aqueous solvent contains a miscible organic diluent, for example a lower alkanol, for example methanol, ethanol, isopropanol or tert.-butanol, or diethylene glycol, triethylene glycol, acetonitrile or the like.

The starting materials for process e) are known or can be prepared according to processes that are known per se, for example by a process described hereinbefore or hereinafter. Reactive derivatives of a cytokine or immunoglobulin containing an aldehyde function can be prepared in accordance with a process as described in European Patent Application 88 695. In this process the carbohydrate residues of the said compounds are converted into aldehyde derivatives by partial oxidation. That Patent Application also describes the formation of compounds having a hydrazine function, for example by the reaction of an amine with p-fluorophenylhydrazine.

Pairs of reactants that can form a carbon-nitrogen single bond in accordance with process f) are, for example, a cytokine containing a free amino group and an immunoglobulin substituted by one or more bridge-formers containing a carbon-halogen bond or an epoxide function, a cytokine substituted by one or more bridge-formers containing a carbon-halogen bond or an epoxide function and an immunoglobulin containing a free amino group, or substituted cytokines and substituted immunoglobulins in which one substituent contains an amino or a hydrazino group and the other substituent contains a carbon-halogen bond or an epoxide func-

tion and in pairs the substituents together form a bridge-former Z. A conjugate of formula I is also formed in process f) from a mixture containing a cytokine, an immunoglobulin and a reactive derivative of a bridge-former containing two carbon-halogen bonds or two epoxide functions.

The nature of the carbon-halogen bond and of the epoxide function is preferably as described above under process d). Reactive derivatives of a bridge-former containing two carbon-halogen bonds are, for example, bis-halomethyl ketones, for example 1,3-dichloro- or 1,3-dibromo-acetone, bis-haloacetic acid derivatives, for example 1,4-butanediyl-bis-iodo- or -bromo-acetate and similar compounds. Reactive derivatives of a bridge-former containing two epoxide functions are, for example, butadiene-bis-epoxide, 1,4-pentadiene-bis-epoxide or bis-glycidyl ether.

The reaction conditions for process f) are the same as the reaction conditions described hereinbefore under process d).

The starting materials for process f) are known or can be prepared according to processes that are known per se, for example by a process as described hereinbefore.

The intermediates and end products of the processes of the invention are, if desired, purified, for example by dialysis against water or buffer solutions, by chromatographic methods, preferably gel chromatography, high pressure liquid chromatography, affinity chromatography or ion exchange chromatography, by ultrafiltration or by ultracentrifugation.

The invention relates also to those forms of the process in which a compound obtainable as intermediate at any stage is used as starting material and the remaining steps are carried out, or the process is discontinued at any stage, or a compound obtainable in accordance with the process of the invention is produced under the process conditions and further processed in situ.

The invention relates also to novel intermediates and processes for the preparation thereof. The starting materials and the reaction conditions are preferably so selected that those compounds referred to in the description as being preferred are obtained.

The conjugates of the invention can be used for the treatment of viral infections and tumours in the form of pharmaceutical preparations that contain an effective amount of the conjugate together with a significant amount of an inorganic or organic, solid or liquid, pharmaceutically acceptable carrier.

Pharmaceutical preparations for parenteral, for example intramuscular, subcutaneous or intravenous, administration to humans are preferred. Such preparations are isotonic aqueous solutions or suspensions containing the active conjugates together with a carrier and, if desired, adjuncts, for example stabilisers, emulsifiers, solubilisers, salts for regulating the pH and the osmotic pressure, preservatives and/or wetting agents. The pharmaceutical preparations can be prepared according to methods that are known per se, for example in a process in which the active conjugates and the pharmaceutically acceptable carriers and adjuncts are mixed, if desired lyophilised, and dissolved before use.

The dosage of the pharmaceutical preparations containing the active conjugates depends on the disorder to be treated, the body weight, age and individual condition of the patient as assessed by the doctor in attendance, and on the method of administration. Compared with the dosage of the corresponding free cytokine, amounts that are from two to approximately one hundred times smaller are used producing the same biological activity. For example, a conjugate containing interferon alpha is administered in from one to three daily dosage units of from 10^4 to 10^6 IU (International Units of interferon activity), preferably from 10^4 to 10^5 IU. The conjugate can also be administered in higher doses, for example in such an amount that the same biological activity is achieved as is customary with free cytokine. As a result, the desired therapeutic effect of the cytokine is achieved with a higher percentage of the treated patients.

The following Examples serve to illustrate the invention but do not limit the scope thereof in any way.

The abbreviations used in the Examples have the following meanings:

IFN interferon
Ig immunoglobulin
IgG immunoglobulin G
IU International Units of IFN activity
PBS phosphate-buffered physiological saline
rIFN recombinant interferon.

Example 1: $[rIFN\alpha_{2b}][(C=NH)CH_2CH_2CH_2SSCH_2CH_2(C=O)][IgG]$

1 mg of polyclonal human antibody IgG (lyophilisate, Intraglobin F^R, Biotest) is dissolved in an Eppendorf tube in 500 μ l of PBS, pH 7.2. 12 μ l of 1.6 mM 3-(2-pyridyldithio)-propionic acid N-hydroxysuccinimide ester and 12 μ l of ethanol are added dropwise to the IgG solution shaken in a Whirlmix and the mixture is incubated at room temperature for 30 minutes. Subsequently the mixture is dialysed overnight against PBS (buffer changed 3 times) at 4°C.

10 μ g of recombinant human interferon α_{2b} are dissolved in an Eppendorf tube in 500 μ l of PBS, pH 7.2. 2-iminothiolane is dissolved at 0°C in 1M triethanolamine hydrochloride, pH 8.0. 5 μ l of this iminothiolane solution are added to the IFN solution and the mixture is incubated at room temperature for 90 minutes. The reaction mixture is dialysed against PBS at 4°C four times, for 1 hour in each case. The IFN solution is mixed with the above IgG solution, shaken at room temperature for 2 hours and maintained at 4°C overnight.

The conjugate is separated from free IFN by ultrafiltration at 4°C using a filter (average exclusion limit 50 000 Daltons). The two fractions are tested for IFN activity in the CPE inhibition assay (Cytopathic Effect, see Ho and Enders, Proc. Natl. Acad. Sci. USA 45, 385-389 (1959)). Active conjugate is found in the supernatant.

For a control, the entire reaction sequence is repeated with radioactively labelled ^{125}I -IFN. Radioactive conjugate is found in fractions 1 to 10, radioactive IFN in fractions 12 to 25.

The same reaction is also carried out with recombinant human interferon $\alpha/\text{B-D}$ hybrid (Ciba-Geigy AG, EP 205 404) and with natural human interferon α (DRK Blutspendedienst [German Red Cross Blood Donor Service] Springe) instead of IFN $_{2b}$.

Example 2: $[\text{IFN}\alpha][(\text{C}=\text{O})\text{CH}_2\text{CH}_2\text{SSCH}_2\text{CH}_2(\text{C}=\text{O})][\text{IgG}]$

2 mg of polyclonal human antibody IgG (lyophilisate, Intraglobin F^R, Biotest) and 100 μg (10^7 IU) of natural human interferon α (DRK Blutspendedienst Springe) are each dissolved in 1 ml of PBS, pH 7.2. 18 μl of 20 mM 3-(2-pyridyldithio)-propionic acid N-hydroxysuccinimide ester and 82 μl of PBS are added to each solution, the mixtures are incubated for 30 minutes at room temperature with gentle shaking, and dialysed overnight at 4°C in each case against 500 ml of PBS. The IFN solution is stabilised with 0.1 % albumin and stored at 4°C. The IgG solution is adjusted to approximately pH 6 with 156 μl of 0.1M sodium acetate (pH 4.5), 3.8 mg of dithiothreitol are added and the whole is incubated for 20 minutes at room temperature then dialysed again overnight at 4°C against PBS. The IFN solution and the thiolated IgG solution are mixed together, shaken for 2 hours at room temperature and maintained at 4°C overnight. The conjugate is then separated off as described in Example 1.

Example 3: Residence time of the IFN-IgG conjugate in murine serum

Three mice are each injected intramuscularly with 0.4 ml of a solution of the conjugate of Example 1 containing 3×10^4 IU. Serum samples are examined for IFN activity in the CPE-inhibition assay after a few minutes, 4, 8, 24, 48, 56 and 80 hours. In this assay the activities measured are as follows (\log_{10} IU per ml):

- 29 -

	0 h	4 h	8 h	24 h	48 h	56 h	80 h
mouse 1	0.5	2.3	2.0	2.1	2.0	2.2	2.0
mouse 2	(2.0)	2.2	2.2	2.2	2.0	2.2	2.2
mouse 3	1.5	2.1	2.2	2.0	2.0	2.2	2.2
Mean value	1.2	2.2	2.1	2.1	2.0	2.2	2.1

By comparison, the IFN activity of intramuscularly injected IFN α_{2b} alone (3 x 10⁴ IU) falls off considerably more sharply:

	0 h	4 h	24 h	32 h	56 h	72 h	80 h
mouse 1	0.7	2.3	0.7	0.5	0.5	0.5	0.5
mouse 2	0.5	2.2	1.6	1.1	0.6	0.5	-
mouse 3	0.5	0.8	2.1	0.9	0.5	0.5	0.5
mouse 4	-	1.7	2.1	1.2	-	0.7	0.5
mean value	0.6	2.0	1.9	1.0	0.5	0.5	0.5

Practically the same values as with IFN α_{2b} alone are found if mixtures of IFN α_{2b} and Intraglobin F (human IgG) are injected.

Example 4: General specification for the preparation of
[cytokine] [(C=O)CH₂CH₂SSCH₂CH₂(C=O)] [Ig]

In each case 2.5 mg of cytokine and immunoglobulin are dissolved in 0.5 ml of PBS pH 7.2 and each solution is incubated for 30 minutes at 25°C with 100 μ l of a freshly prepared 3-(2-pyridyldithio)-propionic acid N-hydroxysuccinimide ester solution (4 mM). A PD-10 gel filtration column is equilibrated with 20 ml of a dithiothreitol solution. The Ig solution is then applied to the column and eluted with 6 ml of acetate buffer of pH 4.5. 500 μ l fractions are collected and the extinction is measured at 280 nm. Protein-containing fractions are combined, applied to a new PD-10 gel filtration column equilibrated with PBS and eluted with phosphate buffer. The cytokine solution is also subjected to gel filtration with phosphate buffer over a PD-10 column, then mixed with the thiolated Ig

solution and incubated for 2 hours at room temperature and for 20 hours at 4°C. The reaction product is analysed using polyacrylamide gel electrophoresis.

Example 5: Pharmaceutical composition

Human albumin is added to solutions containing 10^6 IU of the IFN α conjugate of Example 1 until a final concentration of 1 % is reached, the mixtures are dialysed against PBS at 4°C, sterile-filtered through a bacteriological filter, and the filtrate is filled under aseptic conditions into 10 l ml ampoules. The ampoules are preferably stored at low temperature, for example at -18°C.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:
~~XXXXXXXXXXXXXXXXXXXX~~

1. Conjugates of cytokines with human immunoglobulin.
2. Conjugates according to claim 1 of the formula

Ck-Z-Ig

(1)

in which Ck is a residue of a natural human cytokine or of a recombinant cytokine, Ig is a residue of a human immunoglobulin and Z is a covalent bond or a covalently bonded organic bridge-former, and in which Ck, Ig and/or Z also may occur several times.

3. Conjugates according to claim 2 of the formula I in which Ck is a residue of a natural or recombinant human interferon, of interleukin, of a related compound or of a fragment that retains the biological activity, Ig is a residue of a natural human immunoglobulin, Z is a covalent bond or a covalently bonded di-, tri- or tetra-valent hydrocarbon radical which, if desired, is substituted and/or in which, if desired, one or more carbon atoms have been replaced by oxygen, sulfur and/or unsubstituted or substituted nitrogen atoms, and in which Ck, Ig and/or Z also may occur several times.

4. Conjugates according to claim 2 or 3 of the formula I in which Ck is a residue of a natural or recombinant human interferon or of a related compound, Ig is a residue of a natural human immunoglobulin, and Z is a covalent bond or a covalently bonded divalent hydrocarbon radical which, if desired, is substituted by oxo or imino, and/or in which one or more carbon atoms have been replaced by oxygen, sulfur and/or nitrogen atoms.

5. Conjugates according to any one of claims 2 to 4 of the formula I in which Ck is a residue of a natural or recombinant human interferon alpha or gamma, Ig is a residue of a natural human immunoglobulin G or M, and Z

is a covalent bond, unsubstituted or oxo- or imino-substituted lower alkylene, or combinations of that radical with oxy, thio, amino, phenylene and/or azacycloalkylene.

6. Conjugates according to any one of claims 2 to 5 of the formula I in which Ck is a residue of a natural or recombinant human interferon alpha, Ig is a residue of a natural human immunoglobulin G, and Z is a covalently bonded oxo- and/or imino-substituted lower alkylene-dithio-lower alkylene radical.

7. Conjugates according to any one of claims 2 to 6 of the formula I in which Ck is a residue of recombinant IFN α_2b , Ig is a residue of a natural human immunoglobulin G, and Z is carbonimidoyl-propylene-dithio-ethylene-carbonyl.

8. A process for the preparation of conjugates of cytokines with human immunoglobulin according to claim 1, which comprises reacting a cytokine with human immunoglobulin and, if desired, a bridge-former.

9. A process according to claim 8 for the preparation of conjugates of the formula



which comprises so reacting 2 or 3 reactive sub-units of the conjugate of formula I with one another that a conjugate having covalent bonds is formed, and isolating the conjugate.

10. A process according to claim 9 which comprises:

a) for the preparation of a conjugate of formula I in which the sub-units are linked by an amide bond, condensing a sub-unit containing an amino group with the complementary sub-unit containing a carboxylic acid group or with a reactive derivative thereof, or

- b) for the preparation of a conjugate of formula I in which the sub-units are linked by a disulfide bond, treating two complementary sub-units that both contain a mercapto group with a mild oxidizing reagent, or
- c) for the preparation of a conjugate of formula I in which the sub-units are linked by a disulfide bond, substituting a reactive substitutable functional derivative of the mercapto group of one sub-unit with the complementary sub-unit containing a mercapto group, or
- d) for the preparation of a conjugate of formula I in which the sub-units are linked by a carbon-sulfur bond, adding a sub-unit containing a mercapto group to the complementary sub-unit containing a carbon-carbon double bond or containing an epoxide function, or substituting a carbon-halogen bond by the mercapto group, or
- e) for the preparation of a conjugate of formula I in which the sub-units are linked by a carbon-nitrogen single or double bond, condensing an aldehyde group of one sub-unit with the complementary sub-unit containing an amino or hydrazino group, and, if desired, reducing the resulting carbon-nitrogen double bond to a carbon-nitrogen single bond with a reducing agent, or
- f) for the preparation of a conjugate of formula I in which the sub-units are linked by a carbon-nitrogen single bond, adding a sub-unit containing an amino or a hydrazino group to the complementary sub-unit containing an epoxide function, or substituting a carbon-halogen bond by the amino or hydrazino group.

11. Pharmaceutical preparations containing conjugates of cytokines with human immunoglobulin.

12. A process for the manufacture of pharmaceutical preparations according to claim 11, which comprises mixing conjugates of cytokines with human immunoglobulin with a suitable pharmaceutical carrier.

13. A method of treating viral infections and tumours in an animal including man, which comprises administering an effective amount of conjugates of cytokines with human immunoglobulin according to claim 1.

14. Conjugates of cytokines with human immunoglobulin, substantially as hereinbefore described and exemplified.

15. A process for the preparation of conjugates of cytokines with human immunoglobulin according to claim 1, substantially as hereinbefore described and exemplified.

16. Pharmaceutical preparations containing conjugates of cytokines with human immunoglobulin, substantially as hereinbefore described and exemplified.

17. A process for the manufacture of pharmaceutical preparations according to claim 11, substantially as hereinbefore described and exemplified.

18. A method of treating viral infections and tumours in an animal including man, substantially as hereinbefore described and exemplified.

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DATED this 31st day of August 1988.

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